EFFECTS OF VARIABLES ON BIOPOLYMER PRODUCTION IN 20L BATCH STIRRED TANK FERMENTATIONS

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ABSTRACT

This research is conducted to study the effects of variables on polyhydroxybutyrate(PHB) production in 20L batch stirred tank fermentations by using *Cupriavidus necator*. It consist of three major phases starting with scale up process, followed by fermentation process and mathematical analysis. The fermentation process is scaled up from the optimum condition in 500mL shake flask to 20L stirred tank bioreactor by approximating the set point values of air flowrate and agitation speed which gives the same dissolve oxygen tension (DOT) curve versus time. The closest DOT curve pattern to that of the optimum condition in 500mL shake flask is achieved by the combination 230 RPM agitation speed and 10L/min compressed air flowrate. This experiment is followed with fermentatin process involving the manipulation of the parameters agitation speed, temperature and initial glucose concentration. The 2^3 factorial experiment method is used to design the experiment around the set points of agitation speed and air flowrate which gave the optimum DOT curve. Among the experimental points, it is found that the highest yield of PHB is produced in condition of 210RPM agitation speed, 32°C temperature and 23g/L initial glucose concentration. This experiment was followed by mathematical analysis using Yates' Method to study the main effect and interactive effect of changing the level of experimental variables. The calculation proved that temperature has the biggest main effect on PHB production while the biggest interactive effect is from the combination of agitation speed and initial glucose concentration.

ABSTRAK

Penyelidikan ini dijalankan adalah untuk mengkaji kesan pembolehubah ke atas penghasilan polyhydroxybutyrate(PHB) di dalam tangki penampaian kelompok teraduk dengan menggunakan *Cupriavidus necator*. Ia mengandungi tiga fasa yang dimulai dengan proses menskala naik, diikuti oleh proses penampaian dan analisis matematik. Proses penampaian diskala naik dari persekitaran optimum di dalam kelalang goncang berisipadu 500mL kepada tanki reaktor teraduk berisipadu 20L dengan mendapatkan bentuk lengkuk tekanan oksigen terlarut (DOT) menentang masa yang paling sama. Lengkuk DOT yang paling hampir sama dengan persekitaran optimum di dalam 500mL kelalang goncang diperoleh daripada kombinasi 230 RPM kelajuan pengadukan dan 10L/min kadar aliran udara mampat. Eksperimen ini dikuti oleh proses penampaian yang melibatkan manipulasi pembolehubah kelajuan pengadukan, suhu dan kepekatan awal glukosa. Teknik eksperimen 2^3 faktorial digunakan untuk merekabentuk ekspeiment di sekitar titik kawalan bagi kelajuan pengadukan dan kadar kelajuan udara yang memberikan lengkuk DOT optimum. Daripada kesemua proses eksperiment, didapati bahawa PHB paling banyak dihasilkan dalam persekitaran 210 RPM kelajuan pengadukan, 32°C suhu dan 23g/L kepekatan awal glukosa. Eksperimen ini seterusnya diikuti oleh analisis matematik menggunakan taknik Yates untuk mengkaji kesan utama dan kesan saling tindak akibat perubahan aras pembolehubah eksperiment. Pengiraan menunjukkan bahawa suhu mempunyai kesan utama terbesar terhadap penghasilan PHB manakala kesan saling tindak terbesar pula adalah daripada kombinasi kelajuan pengadukan dan kepekatan awal glukosa

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LIST OF SYMBOLS

PHB	-	Poly-β-hydroxybutyrate
PHA	-	Poly-β-hydroxyalkonoate
DOT	-	Dissolve Oxygen Tension
RPM	-	Rotation Per Minutes
g	-	Gram
L	-	Liter
W%	-	Weight Percent
m	-	Molar
OD	-	Optical Density
mg	-	Miligram
Min	-	Minutes

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CHAPTER 1

INTRODUCTION

1.1 Background Study

In the world that full of fascinating technology and the concern about the facing of the demand, biotechnology is couraged to offer an increasing potential for the production of goods to meet various human needs. The goal of these approach is to meet the criteria sustainability in concept that is introduced by the World Commission on Environment and Development (WCED, 1987). The concept of sustainability aim to promote "..development that meets the needs of the present without compromising the ability of future generation to meet their own needs". To determine the application of the concept, criteria that concern is its economic, environmental and social impact perspective.

In the objective to fulfill the human needs, the plastic is invented from oil and petroleum about in 1933 by German chemist. The plastic that we daily used is the polyethylene plastic which is inexpensive material and be very durable. However, the common property of this polyethylene plastic is the high resistance to natural degradation process and cause a crucial problem to environment in space for landfill (Huisman *et al*.,) So, it is not environmental friendly and contribute to the pollution as the increasing of the solid waste, whose main part is plastic. Furthermore, it is opposite to the concept of sustainable development.

In conjunction to settle this problem, there is the obligation for us to find another alternative material to replace the polyethylene plastic based material that can be easily degrade or biodegradable material in force to save the environment for the next generation. The awareness of this situation lead to the research and invention of biodegradable plastic and environmental friendly. One of them is production of polyhydroxyalkanoates (PHAs) and polyhydroxybutyrate which is microbially-formed. The presence of the polyhydroxybutyrate was confirmed as an energy and carbon source and storage in various bacteria apparently in response to conditions of physiological stress (Kim *et al.,* 1994*)*. The most advantage of PHB is given by its properties that is biodegradable in reasonable time period.

Polyhydroxybutyrate (PHB) is a high molecular weight polyester. It is a biodegradable intracellular microbial thermoplastic that provide carbon and energy reserves in several microorganism. PHB polymer is produced by microorganisms such as *Alcaligenes eutrophus* or *Basillus megaterium* and *Cupriavidus necator* under unfavourable conditions (Lee, 1996). Polyhydroxybutyrate has attracted much commercial interest as a plastic. These polymers have similar properties to some of petrochemical-derived thermoplastics such as polyethylene in term of molecular weight, melting point, stiffness, brittleness and glass transition temperature (Steinbuchel, 2002). PHB polymer is resistant to water and ultravioletradiation and also impermeable to oxygen. Therefore, it is suitable to replace the polyethylene in application for food and drink packaging. Besides, it can help to reduce land pollution due to its characteristic that is biodegradable in soil.

1.2 Problem Statement

Commonly, the production of biopolymer is known as a relatively expensive process especially in large scale or industrial scale compare to the production of polyethylene based material plastic. This is happen due to biological process used that required the usage of growth medium in large scale such as glucose and other mineral which quite expensive, in the other hands, the preparation of the inoculum in the culture media needs 3 days and the extraction process to obtain the biolpolymer or PHB need the usage of specific and expensive solution also contribute to the high cost production process. The cost that required is seems high as it is compared to the concentration of biopolymer that may produce from the fermentation process after several days. Therefore, it is a very strong obligation to optimize the production of bioplastic by study the effect of changing the variables of production and at the same time minimize the cost. Hence it would strengthen the promotion on public to use PHB based material for daily usage.

1.3 Scope of Research Work

The study for effect of variables on biopolymer production in 20L batch stirred tank fermentations by using *Cupriavidus necator* involve the following steps:

- Obtaining from the previous jurnal for optimum condition for the production of PHB of fermentation in 500 mL shake flask.
- Scale up process from 500mL shake flask to 20L fermenter
- The fermentation process in 20L tank fermenter by manipulating the condition of the fermentation.

- Experimental analysis to obtain the production profiles.
- Mathematical analysis to study the effect of variables on production biopolymer in 20L batch stirred tank fermentations.

1.4 Objectives

- To produce polyhydroxybutyrate (PHB) which is biodegradable plastic as a replacement to polyethylene plastic that non-biodegradable.
- To scale-up biopolymer (PHB) fermentation process from scale of 500mL shake flask to scale of 20L stirred tank fermenter.
- To study the effect of variables on production of PHB in scale of 20L stirred tank fermenter by manipulating the parameters which are the agitation speed, initial glucose concentration and temperature of the fermentation process.

CHAPTER 2

LITERATURE REVIEW

2.1 Development of Bioplastic

As the technology skyrocket with new fascinating inventions to fulfill the human demands, a group of materials never seen before on our planet is produced. They were synthetic polymers, also known as plastics. The application of this type of material is widely accepted in daily usage to improve the lifestyle around the world. Contrarily, the wide application of plastic give negative impact on earth due to high resistance of the material to natural degradation process. Statistically, in Malaysia, the per capita generation of solid waste varies from 0.45 to 1.44kg/day depending on the economic status of an area and most of the waste are consist of plastic based material. In general, the per capita generation rate is about 1kg/day. Disposal of solid waste is done solely through landfill method with capacity about 177 disposal sites in Peninsular Malaysia (Malaysia Country Report, 2001).

The usage of the conventional type of plastic, which is polyethylene based material contribute to the huge amount of the solid waste. Hence cause the limitation problem in landfill space due to its non-biodegradable property. Biodegradation process refer to the chemical breakdown of materials by a physiologically environment where the materials are degraded aerobically with oxygen or anaerobically without oxygen. Sometimes, this process is related to the environmental remediation or bioremediation (Diaz E., 2008)

Basically, biodegradable matter is organic material such as plant and animal or other substances originated from living organisms or artificial that similar enough to plant and animal matter to be put to use by microorganisms (Diaz E., 2008). In conjunction to overcome the problem of limitation in landfill space, the production of bioplastic is looked as a precious invention to meet the human demand and at the same time does not give negative effect on the environment. This is due to its biodegradable property compared to the conventional type. In fact, one of the the bioplastic that invented is polyhydroxybutyrate (PHB)

2.2 Polyhpolyhydroxybutyrate (PHB)

2.2.1 Introduction of Polyhpolyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne in the bacterium *Bacillus megaterium*. In subsequent years, it was also found in other species of bacteria as at the end of 1950s, the presence of PHB is confirmed as an a carbon source and energy (Poirier *et al*, 1995). The structure of PHB is shown in the Figure 2.1

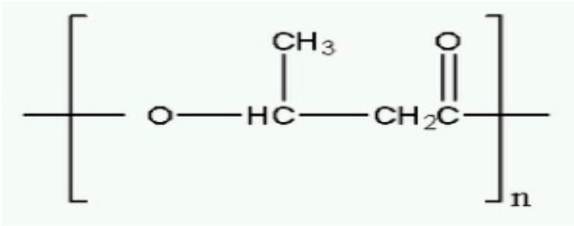

Figure 2.1 Structure of PHB

Bacteria that produce PHB can be divide into two groups. The first one include the *Ralsotnia eutropha (Alcaligenes eutropha),* and consist of bacteria that produce short-chain PHA with C6-C5 monomers, while the second, which contain for example *Pseudomonas oleovorans,* involves microorganisms that produce medium-chain PHA with C6-C14 monomers (Poirier et al., 1995)

PHB is produced by micro-organisms apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by micro-organisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB.

2.2.2 Properties Of Polyhydroxybutrate

Historically, PHB has been studied most extensively and has triggered the commercial interest in this type of polymer. PHB is the common type of PHA , and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic. Copolymers of the PHB can be formed by cofeeding of substrates and may result int the formation of polymer containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers (Brandl *et a*l, 1988).

In general, PHB have the similar properties as the polyethylene plastic with further advantage of PHB for biological degradable and nontoxic material. As addition, PHB have the ability of water insoluble and relatively resistant to hydrolytic degradation which differentiate it with the another biopolymer (Peijs, 2002). The biodegradable ability is the most important characteristic of PHB. In nature, the degradation process depend on the activities of enzyme in the microorganisms. Therefore, the process may vary and depend on the composition of the polymers, its physical form (amorphous or crystalline), the dimensions of the sample and the most important is the environmental conditions (Jendrossek et al., 1998). Typically, the degradation rate of a piece of PHB is around a few month in anaerobic sewage to years in seawater (Mergaert, *et al*, 1995)

Within the cell, PHB exist in a fluid and amorphous state. However, after the extraction process from the cell with organic solvent, PHB becomes highly crystalline and in this state, it is stiff but brittle (Doi, Y., 1995). Besides, the PHB is good oxygen permeability and good ultra-violet resistance but poor resistance to acids and bases. PHB also soluble in chloroform and other chlorinated hydrocarbon and have the ability of biocompatible. It have the melting point of 175°C and glass transition temperature 15°C (Lara L., 1999). PHB has the tensile strength 40 MPa, which close to polypropylene strength. Other physical property of PHB is sinking in water (while polypropylene floats), and cause facilitating its anaerobic biodegradation in sediments.

In the other hands, the production of PHB which is from the fermentation process attract the world attention since the fermentation process is based on the renewable resources and agriculture products such as sugars and fatty acid as the carbon source. Thus, it is looked as the answer to the problem of diminishing fossil fuel stockpiles that used in production of conventional plastic. However, in using the PHB as the plastic material have some disadvantage due to its tendency to be brittle. When it was spun into fibers it behaves as a hard-elastic material (Antipov *et al*, 2006). Besides, the synthesis process of PHB is economically challenging compare to the production of polyethylene plastic.

2.2.3 Application of Polyhydroxybutrate

The main issues that become the driving force the production of PHB is as biodegradable plastic and as replacement to the usege of the non-biodegradable plastics disposal such as polyethylene based material. Recently, the usage of the non-biodegradable plastics may causes significant ecological problems. That is, the availability of landfills is limited and the incineration of plastics increases greenhouse gases and releases toxic compounds. In contrarily, PHB is a biodegradable product, biocompatible thermoplastic and has similar physical properties to polypropylene. It has similar piezoelectric properties to natural bone and is optically active (all of its monomers are the d-isomer).

For further application of PHB, there are numerous research done by extending the physical properties of PHB and the PHA family of polymer by compounding and blending to provide a corresponding broad range of end-use applications. For example is the molding applications in consumer packaging and coating items. Besides, PHB can be used in toner and developer compositions or as the ion-conducting polymers. Instead, PHB also applicable in medicine, veterinary practice and agriculture due to its biodegradability. Its biocompatibility is the reason of medical applications such as surgical pins and sutures.

2.2.4 Metabolic Pathways of PHB synthesis

The biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three distinct enzymes. The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl- CoA by b-ketoacylCoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH- dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB polymerase, encoded by phbC. PHB is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA), acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three-step pathway (Steinbuchel and Fuchtenbusch, 1998). The genes of the phbCAB operon encode the three enzymes. The promoter (P) upstream of phbC transcribes the complete operon (phbCAB) (Daae *et al*, 2008).

2.3 *Cupriavidus necator*

Cupriavidus necator was described as accommodatation of non-obligate bacterial predator of various Gram-negative and Gram-positive soil bacteria and fungi. This organism shared with members of the genus *Alcaligenes*, which, at that time, comprised multiple species, including Alcaligenes faecalis (the type species), *Alcaligenes xylosoxidans* and allied species (now all classified in the genus Achromobacter; and *Alcaligenes eutrophus* (first reclassified in the genus *Ralstonia* and recently transferred again, to the novel genus *Wautersia.*

2.4 Study for Effects of variables

2.4.1 The First Area Of Response Surface Investigation

Once the experimental variables or variables relevant to the fermentation have been screened by using the method of Factor Analysis, the combination of levels of experimental variables that gives the highest yield among other combinations is obtained. Besides, the levels of combination variables can be obtained from scale up process. The combination of levels of variables that gave the highest yield is used as the centre point of first area of response surface investigated for presence of the point of maximum yield. The area around this centre point can be investigated for presence of the point for maximum yield using the method of factorial experiment.

2.4.1.1 The Method of Factorial Experiments

There are many types of factorial experiments, however the common type used is the one that involving two levels or known as the 2^n factorial experiments (Cochran et al., 1957). This method has been designed to allow the effects of a number of experimental variables on the yield to be investigated simultaneously.

This method gives the ‘main effects’ and the ‘interactive effects’ of changing the levels of the experimental variables from the lower level to the upper level. The main effect of an experimental variable is defined as the average of the effect of changing its value from the lower level to the upper level among all experiments. It is derived by assuming that the experimental variable is an independent parameter and all variations in its effects are due to experimental only.The interactive effects between two or more experimental variables are calculated on the assumption that the experimental parameters are not independent but are in fact interacting between them.

2.4.1.2 Yates’ Method

This mathematical method is used to analyze the main effects and interactive effects. At the same time, the result that obtained from the calculation of this method also indicate whether the yield response surface in the area of examined is curved or incurved, whether it is flat or not, whether increasing or decreasing with relation to one or more experimental variables and in which direction.

CHAPTER 3

METHODOLOGY

3.1 Research Background

The study effects of variables on production in 20L batch stirred tank fermentations is carried out with 15L working volume by using microorganism *Cupriavidus necator*, formerly known as *Ralstonia eutropha* to produce PHB which is the biopolymer.

Previously, the optimization process of production PHB already carried out in small scale fermentation process which is conducted in 500mL shake flask with 200mL of working volume. Project is extended by optimizing the production of PHB in fermentation process that carried out in big scale of 20L stirred tank fermenter with 15L working volume. The research consists 6 phase of experimental and mathematical methodology which are:

i. Obtaining the information from previous research, (Aisyah Azmi, 2009)
ii. Scale up process.

iii. Mathematical method of Factorial experiment.

iv. Fermentation.

v. Sample analysis.

vi. Mathematical method for optimization process.

3.2 Materials

In this experiment, the material that used are microorganism *Cupriavidus necator;* mineral agar medium for cultivation of microbe, growth medium, glucose as the carbon source, nitrogen gas and compress air.

3.3 Previous Research

The highest concentration of PHB that produce at the optimum parameter is 1.0802 g/L with 5.8350 g/L biomass. The optimum values of parameters are 28.46 °C, initial glucose concentration of 28.66 g/L, agitation speed of 251.93 RPM and initial peptone concentration of 10.05 g/L. Besides, the journal also show that the main effect of the initial concentration of peptone give low effect on the production of PHB and can be negligible compare to another parameters. Therefore, in this extending research, the parameters that manipulated are agitation speed of impeller, temperature of fermentation and initial concentration of glucose(Azmi, A.,2009)

3.4 Scale Up Process

Scale up process is carried out to obtain similar amount of product per volume for small scale and large scale in the same time duration. The main part in scale up process is by maintaining the rate of oxygen transfer in the large scale like already achieve in the small scale. The method for scale up process that used is applied in this is known as Gassing Out Technique is used to obtain the dissolve oxygen transfer (DOT) curve in small scale of shake flask and in 20L fermenter (Salihon, J., 1995)

An oxygen probe is connected to the oxygen meter and immersed in a 500mL shake flask that filled with 200mL of distilled water. Nitrogen gas is sparged into the flask until the reading of oxygen probe show zero. Calibration method is required as the reading of oxygen probe unable to achieve value of zero. After that, the flask is shaked in the orbital shaker with the speed of 250 RPM and air flowrate of 1L/min. The values that used are the optimum value of parameters for production PHB in 500mL shake flask which already obtained by the previous research. At the same time, the reading of the oxygen probe is taken for each minutes. Graphically, the oxygen probe reading versus time is plotted.

The steps above is repeated by using the scale of 20L stirred tank fermenter with 15L of working volume. This equipment is filled with distilled water to obtain the same pattern of DOT curve as already obtained in 500mL shake flask. The values of the agitation speed and the air flowrate are determined by using trial-and-error technique. The values of parameter that obtain is used in the next step of research.

3.5 Mathematical Method of Factorial Experiment

This method is used to determine the number of experiments that should carried out in order to investigate the effect of three parameters simultaneously. The mathematical equation that used is shown as below:

Number of experiment = 2^n n= 3 parameters used.

Therefore,

number of experiment= 2^3 = 8

From the calculation, the table that show the combination of the parameter are constructed as shown in table below:

Table 3.1 Experimental Table Constructed

Number	Agitation Speed (RPM)	Temperature (°C)	Glucose Concentration (g/L)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1